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SOME NITROGENOUS CONSTITUENTS OF CORN POLLEN

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SOME NITROGENOUS CONSTITUENTS OF CORN POLLEN[1]

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HISTORICAL

The first work recorded on the chemical analysis of pollen was by Fourcroy and Vauquelin (*1*),[2] who studied the ash and organic constituents of pollen of the date palm, *Phoenix dactylifera*. They found that this pollen contained a substance analogous to gluten, and a large amount of malic acid. Link (*9, p. 145*) reported much tannin and gluten in the pollen of the hazel tree. Vauquelin (*9, p. 237*) found sugar, resin, and tannin in stamens of the horse chestnut. Braconnot (*6*) reported the presence of potassium malate and a little nitrogenous material in the pollen of *Typha latifolia*.

Planta (*35*) found sucrose, starch, and 4.81 per cent nitrogen in the pollen of *Corylus avellana*. An extract of this pollen with 10 per cent NaCl solution gave a coagulum on heating, and peptones were also present. In the pollen of *Pinus sylvestris*, Planta (*36*) found 2.65 per cent nitrogen. Peptones, guanine, and hypoxanthine were present. An extract of the pollen with 10 per cent NaCl solution did not, however, give a coagulum on heating. Schulze and Planta (*39*) found a nitrogenous substance in the pollen of Corylus and pine. This substance yielded guanine and glucose, hence was called guanosin. Przybytek and Famintzin (*37*) found 2.4 per cent nitrogen in the pollen of *Pinus sylvestris*, and Kresling (*25*) reported tartaric and malic acid in the same kind of pollen.

Stift (*41*), working on pollen of the sugar beet, found the total nitrogen to be 3.6 per cent, protein nitrogen 2.66 per cent, and lecithin 1.57 per cent. Stoklasa (*42*) reported 5.16 per cent lecithin in pollen of the horse chestnut, and 5.86 per cent in that of the apple. Kammann (*21*) found that nitrogen containing organic substances made up 58 per cent of the total organic material of rye pollen, and of this 40 per cent was protein in nature and 18 per cent nonprotein. Winterstein and Hiestand (*50*) found in the pollen of *Alnus viridis* and *Pinus montana* a phosphatide which contained lecithin in combination with a complex carbohydrate.

Koessler (*24*) found the following nitrogen distribution in ragweed pollen:

	Per cent
Total nitrogen	4. 73
Nitrogen soluble in 5 per cent NaCl solution	1. 89
Nitrogen soluble in 5 per cent NaCl solution and coagulable	. 10
Nitrogen soluble in 5 per cent NaCl solution and noncoagulable	1. 66
Nitrogen soluble in 75 per cent alcohol after 5 per cent NaCl extraction	1. 57
Nitrogen in the insoluble residue	1. 35

[1] Received for publication Mar. 14, 1927; issued September, 1927. Published with the approval of the director as Paper No. 675, Journal Series, Minnesota Agricultural Experiment Station. Condensed from a thesis presented by C. G. Vinson to the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of doctor of philosophy, Ross Aiken Gortner, major adviser.

[2] Reference is made by number (italic) to "Literature cited," p. 275.

Heyl has done the most extensive work on the chemical analysis of pollen. He found (*13*) that the exine was removed largely by extracting with ether and then alcohol; but that this did not remove the intine which is largely cellulose. Extracting with ether in a Soxhlet apparatus removed 13.26 per cent of the solids, and alcohol following the ether extraction removed an additional 20.9 per cent; but hot 95 per cent alcohol alone extracted 42.9 per cent of the solids. The pollen contained 4.99 per cent nitrogen, of which 1.08 per cent was alcohol soluble, 2.6 per cent was soluble in 10 per cent NaCl solution, and 0.8 to 1.1 per cent was extracted by 0.2 per cent alkali after extracting with 10 per cent NaCl. The residue from the various extractions contained 1.2 per cent nitrogen. In other papers Heyl (*16*, *17*) reports the nonnitrogenous constituents extracted by alcohol and ether. Ragweed pollen (*14*), which had been exhausted with ether and 95 per cent alcohol and then extracted with distilled water, gave 1.22 per cent albumin, coagulating at 65° C., and 1.1 per cent proteose. After extracting with distilled water and then with 10 per cent NaCl, dilute alkali extracted a protein which amounted to 2.9 per cent of the original material. This protein was precipitated on acidification, and was the chief protein present. The percentage of certain amino acids in these proteins has been reported by Heyl and Hopkins (*18*). Heyl (*15*) found that the coloring substances of ragweed pollen belong to the flavonols and are entirely glucosidic. Quercetin glucoside having the composition of $C_{21}H_{20}O_{12}$ was found. A glucoside of isorhamnetin was also obtained.

Corn pollen, however, has not received so much attention as ragweed pollen. Miyake (*28*) found phytosterol and inosite in corn pollen. Miyake (*29*) later identified adenine and choline. Anderson and Kulp (*3*), working on corn pollen, found 4.3 per cent nitrogen in the air-dry pollen. Phytosterol palmitate was identified. Inosite was found to be present to the extent of 0.83 per cent, choline 0.34 per cent, and l-proline to the extent of 0.6 per cent.

Anderson (*2*) found a saturated hydrocarbon, probably nonakosane, attached to the phytosterol palmitate from corn pollen. The phytosterol preparations differed, however, from ordinary phytosterol.

MATERIAL

Since no particular attempt had been made to identify the nitrogenous constituents of corn pollen, it was decided to concentrate on the nitrogenous fraction. About 4 kgm. of air-dry pollen, collected in 1917 and 1918, were available. The pollen was quite clean, as it had been screened through bolting-cloth, thus freeing it from anther sacs, filaments, etc. The air-dry pollen had a nitrogen content of 3.6 per cent and a moisture content of 3.97 per cent, as determined by bringing a sample to constant weight at room temperature in vacuo over H_2SO_4, as did Anderson and Kulp (*3*). Corn belongs to the anemophilous class of pollinated plants, and agrees with the findings of Lidforss (*26*) in that the nitrogen content of its pollen is relatively low as compared with pollen of entomophilous flowers.

According to Heyl (*14*), the walls (exine and intine) of the pollen grain constitute 65 per cent of the structure. The exine is relatively thick and resistant to mechanical disintegration. Planta (*35*) found that grinding pollen between grooved steel plates had no effect on

the grains. Winterstein and Hiestand (*50*) state that it is difficult to extract the entire grains, so part was crushed in a mortar and part ground in a mill. Anderson and Kulp (*3*) found that long grinding in a mortar, even after the pollen had been extracted with ether and alcohol, produces only a small percentage of broken cells. Realizing that a better extraction could probably be obtained with the pollen grains pulverized, the writer placed 313 gm. of the air-dry pollen in a ball mill of 4,000 c. c. capacity, and at the end of 24 hours grinding not an entire pollen grain could be found when a sample was examined under the microscope. After the ball mill had proved so efficient in pulverizing the grains, practically all the pollen was put through it.

PRELIMINARY EXPERIMENTS

Ten grams each of the milled and unmilled pollen were extracted with two 75 c. c. portions of ether in a Soxhlet apparatus, allowing 12 hours to each extraction. This was followed by extraction with two 150 c. c. portions of absolute alcohol in a 300 c. c. Florence flask, allowing 12 hours to each extraction. From the milled sample 3.0200 gm. of dry matter containing 0.0300 gm. of nitrogen were extracted, and from the unmilled 3.0250 gm. containing 0.0330 gm. of nitrogen, indicating that the unground pollen was as readily extracted by ether and alcohol as was the ground material.

Again 10 gm. each of fresh milled and unmilled pollen were extracted 14 hours in the cold with 150 c. c. of 5 per cent K_2SO_4 solution. The filtered extracts were made up to volume and total nitrogen determined on aliquots. The extract from the unmilled pollen contained 0.1455 gm. of nitrogen and that from the milled sample contained 0.1765 gm. of nitrogen, showing that the nitrogen was more readily extracted from the milled than the unmilled pollen. Approximately 50 per cent of the nitrogen was extracted from the milled pollen by the K_2SO_4 solution.

The residue of the unmilled pollen from the above extraction with 5 per cent K_2SO_4 solution was extracted 15 hours at room temperature with 150 c. c. of 5 per cent H_2SO_4. After filtering and making the filtrate up to volume, it was found to contain 0.0525 gm. of nitrogen. The residue from this extraction was then treated with 150 c. c. of 0.2 per cent NaOH, but after mixing, the reaction was neutral to litmus. Accordingly, sufficient additional dilute NaOH solution was added to give an alkaline reaction to litmus. After extracting for 15 hours the material was filtered by suction, washed, and the filtrate made up to volume. The total nitrogen in this extract was found to be 0.0512 gm. Thus the total nitrogen successively extracted from a sample of unmilled pollen by 5 per cent K_2SO_4, 5 per cent H_2SO_4, and then dilute alkali was $0.1455+0.0525+0.0512=0.2492$ gm. This is 2.49 per cent of the original air-dry pollen; hence the total nitrogen not extractable with aqueous solutions $=3.60-2.49=1.11$ per cent. Heyl (*13*) found 1.2 per cent nitrogen in the residue of ragweed pollen from similar extractions.

The residue from each of the above samples which had been extracted with ether and boiling absolute alcohol was extracted in the cold with 150 c. c. of 5 per cent K_2SO_4 solution for 14 hours. On filtering and making the filtrates up to volume, that from the milled

sample was found to contain only 0.036 gm. of nitrogen and that from the unmilled sample 0.046 gm. Apparently the ether and boiling alcohol made the remaining nitrogen less soluble, and the more intimate contact of the ether and alcohol with the ground pollen rendered a greater amount of the remaining nitrogen insoluble than in the case of the unground pollen. This precluded the possibility of extracting the pollen with ether and boiling alcohol before extracting the nitrogenous fractions with aqueous solutions. Heyl (*14*), however, found that in the case of ragweed pollen exhaustion with ether and alcohol at ordinary temperatures did not render the remaining nitrogen less soluble.

Ten gm. of milled pollen were extracted 24 hours with 150 c. c. of distilled water, the pollen residue centrifuged off, and the supernatant liquid then filtered. The pollen residue was then treated with 75 c. c. of distilled water, centrifuged, and the washings filtered. The filtrates were combined, made up to volume, and found to contain 0.129 gm. of nitrogen. The 5 per cent K_2SO_4 extraction therefore removed $0.1765 - 0.1290 = 0.0475$ gm. more of nitrogen than the distilled water alone. Therefore, to determine if a globulin was present, one 10-gram sample of milled pollen was extracted 14 hours with 100 c. c. of distilled water and another 10-gram sample of the milled pollen was extracted for a like period with 5 per cent K_2SO_4. The extracts were centrifuged and then filtered. Each was gradually heated. An opalescence began to form at 75° C. and a slight coagulum appeared at 80°. On heating each to boiling under a reflux for an hour, then filtering, 0.0346 gm. of coagulum was obtained from the aqueous extract and 0.0556 from the 5 per cent K_2SO_4 extract. Five gm. of K_2SO_4 were then added to the aqueous extract and the solution again heated to boiling under a reflux for one hour. Another coagulum weighing 0.0294 gm. was obtained, making a total of 0.063 gm. of coagulum from the distilled water extract, which coagulum contained 0.0056 gm. of nitrogen, while that from the 5 per cent K_2SO_4 extract contained 0.0053 gm. of nitrogen. The distilled water had, therefore, removed as much of the heat-coagulable nitrogenous material as the 5 per cent K_2SO_4, and, therefore, the presence of a globulin was improbable, since the salt content of the pollen would hardly give a high enough concentration to hold globulins in solution. Heyl (*14*) found, however, that salt extraction removed more of the heat coagulable fraction from ragweed pollen than extraction with distilled water.

ENZYMES

On warm days when the hand came in contact with the pollen the small amount of perspiration on the fingers seemed to cause the pollen to partially autolyze and form a waxy paste. If this was a case of autolysis, then enzymes must have been present. Kammann (*22*) found proteose in rye pollen. Paton (*32*) working on the pollen of Easter lily, red maple, Norway maple, Siberian crab apple, Austrian pine, magnolia, dandelion, goldenrod, ragweed, and corn, found erepsin, pepsin, and trypsin in some. Trypsin and pepsin (*33*) were found in corn pollen. Hence it was quite necessary to determine whether proteolytic enzymes were present before attempting to isolate the nitrogenous fractions. In order to try to determine the presence of active enzymes, ten 5-gram samples of

the milled pollen were placed in 10 separate 250 c. c. Erlenmeyer flasks, and the flasks were then plugged with cotton and allowed to stand in an incubator at 35° C. overnight. The next morning 100 c. c. of distilled water, which had attained the same temperature by standing in the incubator overnight, were added to each of the 10 flasks, rotating the flasks to secure thorough wetting of the pollen, and 2 c. c. of toluene were added. The flasks were then agitated at half-hour intervals, except at night. The water was added at 7.30 a. m. The first flask was removed at 8 a. m.; the second at 10 a. m.; the third at 12.30 p. m.; the fourth at 3 p. m.; the fifth at 7 p. m.; the sixth at 8 a. m. the second day; the seventh at 4 p. m. the second day; the eighth at 8 a. m. the third day; the ninth at 8 a. m. the fourth day; and the tenth at 8 a. m. the fifth day. On being removed from the incubator each sample was made up to 150 c. c., 50 c. c. of which were used for the $SnCl_2$ precipitation [3] and another 50 c. c. for the copper precipitation.[3] After precipitating the proteins in the two latter fractions with $SnCl_2$ and copper reagents, respectively, each was made up to 100 c. c., centrifuged, filtered, and total nitrogen determined on 25 c. c. samples and amino nitrogen determined on 2 c. c. samples. The 50 c. c. remaining, after taking the samples for $SnCl_2$ and copper precipitation, were filtered, and total nitrogen was determined on 25 c. c. samples and reducing sugars on 5 c. c. samples. Table 1 shows the data which were obtained.

TABLE 1.—*Autolysis of corn pollen (5-gram samples)*

Sample No.	Total nitrogen in the entire aqueous extract	Total nitrogen not precipitated by $SnCl_2$	Total amino nitrogen not precipitated by $SnCl_2$	Total nitrogen not precipitated by copper	Total amino nitrogen not precipitated by copper	Total reducing sugars
	Grams	*Grams*	*Grams*	*Grams*	*Grams*	*Milligrams*
1	0.055	0.025	0.002	0.020	0.008	48.76
2	.056	.032	.006	.025	.006	65.76
3	.056	.036	.003	.036	.002	72.75
4	.059	.027	.000	.031	.000	77.51
5	.066	.027	.002	.039	.002	77.83
6	.064	.034	.005	.039	.001	80.05
7	.063	.025	.005	.043	.006	82.50
8	.062	.037	.006	.044	.004	85.77
9	.071	.033	.016	.039	.010	88.31
10	.075	.036	.016	.043	.016	91.17

The precipitation with copper as recommended by Olsen [4] was much less satisfactory than with the $SnCl_2$ reagent, owing to the fact that the end point with phenolphthalein was obscured by the color of the solution. The same difficulty was also experienced in using bromcresol purple as an indicator in the $SnCl_2$ precipitation.

The small amount of amino nitrogen present renders these determinations subject to great error, and perhaps of little value. It is evident, however, that the total nitrogen in the filtrates from the two precipitations did not increase to any appreciable extent after

[3] OLSEN, A. G. A STUDY OF THE PROTEASES OF BREAD YEAST, SACCHAROMYCES CEREVISIAE. [Unpublished master's thesis. Copy on file, Div. Agr. Biochem., Univ. Minn., St. Paul.] Part of the data in this thesis has been published (*31*).

[4] Olsen, A. G. Op. cit.

the first two hours. The total reducing sugars, however, show a steady increase, as might be expected from the reports of Van Tieghem (*45*), Erlenmeyer (*10*), and Green (*12*), working on the pollens of other species; hence carbohydrases were present and active.

From the above data it was evident that extractions of the pollen could be made with aqueous solutions without danger of alteration of the nitrogenous fractions by proteolytic enzymes.

PRELIMINARY SURVEY

For preliminary survey work, 500 gm. of the milled pollen were extracted 20 hours with 3,000 c. c. of distilled water. Fifteen hundred cubic centimeters of the water were first added, but after agitation and standing 15 minutes the entire mass set to quite a stiff gel. Davison (unpublished data) later found a very active pectase in this sample of pollen, which may explain the gel formation. Lloyd (*27*) has shown that the pollen tubes may show great variation in the amount of water which they will absorb. This may be associated with a gel formation.

The aqueous extraction was carried out by keeping the material, which was in a 5-liter bottle, on a shaking machine for eight hours. The remainder of the time the bottle was shaken at intervals. At the end of 20 hours the material was passed through a supercentrifuge. The filtrate was then filtered through paper pulp in order to clarify it. The solution was then carefully concentrated in vacuo, keeping the temperature below 50° C. This solution was acid to Congo red in reaction, and on attempting to coagulate the heat-coagulable proteins by boiling, only a slight turbidity developed. On testing for albumin by the method of Sörensen (*40, p. 28*) the turbidity increased only slightly. This indicated the presence of very little albumin in the corn pollen. Heyl (*14*), however, found 1.22 per cent of albumin in the aqueous extract of ragweed pollen.

The pollen residue from the above aqueous extraction was extracted with 2,000 c. c. of 0.2 per cent NaOH solution for 24 hours, being kept on a shaking machine for eight hours. This material was likewise put through the supercentrifuge, as it was impossible to filter by gravity or suction. The ordinary centrifuge could not be used, as much of the fine material could not be thrown down with this type of centrifuge. The filtrate was dark in color and quite viscous. It was filtered, and a gelatinous precipitate filtered off. The reaction of the filtrate to litmus was neutral; so evidently not enough alkali had been used to keep the material in solution. The gelatinous precipitate was extracted with 3,000 c. c. of a 0.4 per cent NaOH solution, and on filtering only a negligible residue remained. The filtrate was very dark in color and on being made acid to litmus, but alkaline to Congo red, by carefully adding 5 $N.H_2SO_4$ a gray-colored flocculent precipitate settled out, leaving a clear amber-colored supernatant liquid.

The gray precipitate was extracted 24 hours at room temperature with 1,000 c. c. of 5 per cent K_2SO_4 solution, remaining on the shaking machine eight hours. The solution was filtered, and the filtrate plus the washings concentrated and made up to 1,000 c. c. It was found to contain about 0.100 gm. of nitrogen. If this latter extract represented globulin, certainly there was very little present in the pollen worked with. Heyl (*14*) found an albumin in ragweed pollen,

but does not report the presence of globulins. Bauman, Chudnoff, and Mackenzie (*5*), however, speak of the globulin fraction. The precipitate obtained on neutralizing the alkaline extract is termed the glutelin fraction.

MAIN EXPERIMENT

OUTLINE OF PROCEDURE

One kilogram of the ground pollen was placed in a 5-liter bottle and 3,000 c. c. of distilled water added. In using 0.2 per cent NaOH solution on the preliminary sample, the resulting solution was neutral to litmus; consequently, in this case, 15 per cent NaOH solution was added until the mixture gave an alkaline reaction to litmus, 200 c. c. of the alkali solution being required. The pollen was extracted 24 hours, being kept on a shaking machine for 10 hours. A like extraction was made of another kilogram sample, and this plus the 500 gm. used in the preliminary work made 2½ kgm. extracted. At the end of each extraction the material was passed through a supercentrifuge, and then the filtrates were combined. The filtrate from the above extractions was alkaline to litmus, but not to phenolphthalein. The pollen residue when suspended in 3,000 c. c. of distilled water again gave a neutral reaction to litmus. This suspension was made alkaline to litmus with NaOH solution, and allowed to stand 14 hours with shaking at intervals. At the end of the extraction the material was centrifuged as before.

Upon adding the second extract to the first the combined extracts gave an alkaline reaction to phenolphthalein. By carefully adding $5N.H_2SO_4$ the reaction was carried to that point where all material was yet in solution but precipitation would take place with only a slight amount of acid. The solution was then concentrated in vacuo at a very low temperature. The solution (No. 11) was made up to 8,000 c. c. and the following data obtained from its analysis:

	Grams
Total nitrogen	51.412
Ammonia nitrogen	.440
Amide nitrogen	1.840
Amino nitrogen	8.444
Organic solids	816.64

After aliquoting, $5N.H_2SO_4$ was slowly added to the remainder of the concentrated extract until it was just acid to Congo red, when a gray-colored precipitate formed. This precipitate was light, and tended to come to the top of the liquid. By adding ether and rapidly stirring the liquid it was found that the precipitate would form a uniform layer at the top from which it could be siphoned off into ordinary centrifuge tubes and a further separation obtained by centrifuging in the ordinary type of centrifuge.

After separating the glutelin precipitate in this fashion it was extracted with 2 liters of ether, to remove fat-soluble materials. The precipitate was then dissolved in 6 liters of slightly alkaline water, and reprecipitated by acidifying with acetic acid. The dry precipitate weighed 248.1 gm., contained 22.461 gm. of nitrogen and 19.97 gm. of ash.

The combined filtrates from the glutelin precipitation were concentrated in vacuo and extracted with 2 liters of ether. The aqueous solution was then concentrated, made up to volume (solution 12), aliquoted, and the following data obtained on analysis:

	Grams	Per cent of solution 11
Total nitrogen	25.790	52.68
Ammonia nitrogen	.320	72.82
Amide nitrogen	.721	39.18
Amino nitrogen	7.372	83.71
Organic solids	716.400	87.73

As one would expect, the greater amount of the amino nitrogen came down in the filtrate from the glutelin, and by difference the greater amount of the amide nitrogen remained in the glutelin fraction.

The remainder of solution 12 was hydrolyzed 24 hours with 20 per cent H_2SO_4. The humin from this hydrolysis weighed 49.22 gm. and contained 1.942 gm. of nitrogen. Sulphate was exactly removed with $Ba(OH)_2$. After precipitation was complete the solution containing the $BaSO_4$ was heated and kept almost at the boiling point for about two hours, then allowed to cool slowly. In this way the $BaSO_4$ precipitated in relatively coarse particles, and the solution then filtered fairly readily and a relatively small amount of nitrogen was lost by adsorption. The $BaSO_4$ weighed, when dry, 3,712 gm. and contained 0.989 gm. of nitrogen, which is a low figure for nitrogen in such a large amount of precipitate.

The filtrate and washings from the $BaSO_4$ precipitate were concentrated, made up to volume (solution 13), aliquoted, and on analysis, gave the following:

	Grams
Total nitrogen	25. 270
Ammonia nitrogen	1. 477
Amino nitrogen	10. 351
Organic solids	602. 190

PRECIPITATION WITH NEUBERG AND KERB'S REAGENT

The remainder of solution 13 was concentrated, placed in a large precipitating jar, and precipitation with Neuberg and Kerb's (*30*) reagent carried out. Solutions containing 600 gm. of mercuric acetate and 215 gm. of Na_2CO_3 were added until the volume reached 6 liters and no end point was indicated. It was then considered advisable to add an equal volume of alcohol, remove the precipitate, acidify and concentrate the filtrate, and repeat the Neuberg precipitation at greater concentration, as this reagent is supposed to act best when the amino acid concentration is 10 to 20 per cent. In the second precipitation 195 gm. of mercuric acetate and 60 gm. of Na_2CO_3 were added, and still no orange-red precipitate was obtained on adding either of the two reagents. The failure to observe the characteristic colored precipitate at the end point may have been due to the color of the solution obscuring it, as in the first precipitation an orange-red precipitate collected on the stirring rod, and the last precipitate had a yellow-red layer on top. The last precipitate was very small. Both precipitations were carried out in the cold, the precipitating jar being surrounded by running water which was at a temperature of 10° C. In each case after adding the Neuberg reagent an equal volume of alcohol was added and the mixture then allowed to stand overnight. The two precipitates were combined and washed with 1,000 c. c. of 80 per cent alcohol. On acidifying the filtrate from the first precipitate with acetic acid, a flocculent precipitate formed which was filtered off and was found to be crystalline. It weighed 36.453 gm. and had a distinctly bright, metallic luster. It contained neither nitrogen nor sulphur. This substance is reserved for further investigation.

The filtrate from the last Neuberg precipitate was acidified, concentrated, and mercury was then removed as HgS. The filtrate and washings from the HgS were concentrated and NaCl removed

with concentrated HCl and alcohol. The filtrate from the NaCl was concentrated and made up to volume (solution 15), aliquoted, and the following figures obtained on analysis:

	Grams
Total nitrogen	11. 127
Ammonia nitrogen	. 669
Amino nitrogen	2. 164
Organic solids	266. 325

PRECIPITATION OF BASES FROM THE NEUBERG FILTRATE

Bases were precipitated from the remainder of solution 15 by adding 5 per cent by weight of HCl and then adding a 20 per cent solution of phosphotungstic acid in 5 per cent HCl until precipitation was complete. After standing in the cold overnight, the precipitate was filtered off, and washed with a 2.5 per cent solution of phosphotungstic acid in 5 per cent HCl. Phosphotungstic acid was then removed from the basic precipitate with amyl alcohol and ether according to the method of Van Slyke (*44*) and Jacobs (*20*). The precipitate was suspended in water in a large Erlenmeyer flask, acidified with concentrated HCl, a mixture of equal parts of amyl alcohol and ether was added, and the flask agitated. It was necessary to filter twice before obtaining a good boundary between the two liquids the first time. Three extractions with the amyl alcohol–ether mixture were sufficient to remove all phosphotungstic acid from the aqueous solution so that no precipitate was obtained on adding a drop of a saturated solution of $Ba(OH)_2$. A small amount of the precipitate failed to go into solution. This was ground up with baryta and water and yet failed to go into solution. The alkaline extract thus obtained was very dark in color, and on being acidified gave a precipitate of colored material. It contained a total of 0.082 gm. of nitrogen. It is believed the insoluble part was largely humin.

BASES OF THE NEUBERG FILTRATE

The basic fraction as precipitated from the Neuberg filtrate (solution 24) with phosphotungstic acid contained 3.587 gm. of nitrogen, 0.2989 gm. of which was animo nitrogen, showing that only about 14 per cent of the amino nitrogen of the Neuberg filtrate was basic nitrogen. Schulze's (*38*) method was accordingly used to separate choline from other plant bases. Mercuric chloride in boiling aqueous solution was added to the remaining portion of solution 24 until no further precipitate was obtained (*4*), and the mixture was allowed to stand overnight in the cold before filtering. The precipitate was extracted with boiling water, the insoluble portion was filtered off, and the two filtrates were combined. Mercury was removed as HgS, and alcohol was added to the concentrated filtrate from the HgS. A precipitate weighing 8.849 gm. and containing 0.2159 gm. of nitrogen formed at this point. This consisted of a mixture of ammonium and sodium chloride. The filtrate from the above precipitate was made up to volume and on analysis was found to contain 1.825 gm. of nitrogen. To an aliquot of this solution a 5 per cent platinic chloride solution was added until precipitation was complete. The chloroplatinate obtained weighed 0.4538 gm. and contained 31.84 per cent platinum; hence it must have been choline chloroplatinate.

The insoluble mercury compound was suspended in water and decomposed with H_2S. The filtrate from the HgS was taken down to dryness on a water bath. The residue was dissolved in a little hot absolute alcohol and again concentrated to dryness in vacuo. Very large needle-shaped crystals formed. The crystals were dissolved in absolute alcohol with warming and made up to 250 c. c. (solution 33). This solution contained 1.005 gms. of nitrogen. To 50 c. c. of this solution platinic chloride was added until precipitation was complete. A total of 2.222 gm. of chloroplatinate were obtained from this aliquot, thus giving a total chloroplatinate obtainable from the solution of 11.110 gm. This chloroplatinate was found to contain 31.73 per cent of platinum; hence it must have been choline chloroplatinate as the theoretical platinum content for choline chloroplatinate is 31.68 per cent. From the betaine fraction, as precipitated from the Neuberg filtrate, a total of 13.379 gms. of choline chloroplatinate were thus obtainable, representing 0.608 gm. of nitrogen.

THE NEUBERG PRECIPITATE

The Neuberg precipitate was brought into suspension in water, acidified with acetic acid, and decomposed with H_2S. The filtrate and washings from the HgS were concentrated and made up to volume (solution 14), aliquoted, and on analysis found to contain:

	Grams
Total nitrogen	11. 810
Ammonia nitrogen	2. 058
Amino nitrogen	3. 980
Organic solids	95. 720

Following the use of Neuberg's reagent the ammonia nitrogen increased 84.63 per cent over that in solution 13, and the amino nitrogen decreased 40.64 per cent. In some similar work reported by Vickery and Vinson (*48*), following the use of Neuberg's reagent, the ammonia nitrogen increased 7.3 per cent and the amino nitrogen decreased 15.53 per cent. It is entirely possible that the poor results in precipitating amino nitrogen with Neuberg's reagent were due to adding an excess of the reagent; for mercury is known to be a very effective catalyst, and an excess of the mercuric acetate may bring about a decrease in the amino nitrogen by catalyzing deamination and ring formation.

BASIC FRACTION OF THE NEUBERG PRECIPITATE

Bases were precipitated by phosphotungstic acid from the fraction precipitated by Neuberg's reagent. The filtrate from the basic fraction, after the removal of phosphotungstic acid with $Ba(OH)_2$ and subsequent removal of the barium with H_2SO_4, was found to contain 4.292 gm. of nitrogen, 2.811 gm. of which were amino nitrogen. On finding the ratio of amino to total nitrogen so low, the solution was again hydrolyzed for 10 hours with 10 per cent by weight of H_2SO_4. After removing the H_2SO_4 quantitatively the solution was found to contain 4.292 gm. of nitrogen, 3.505 gm. of which were amino nitrogen; thus, due to hydrolysis, the amino nitrogen was increased 24.7 per cent. Bases were again precipitated, and the precipitate obtained added to the main phosphotungstate precipitate, which was then decomposed with $Ba(OH)_2$. The filtrate from the barium phos-

photungstate precipitate, after removal of barium was found to contain:

	Grams
Total nitrogen	3. 274
Ammonia nitrogen	. 291
Amino nitrogen	1. 133
Organic solids	20. 610

The ammonia nitrogen in the above was probably a volatile amine, in part at least, for in titrating the distillate after ammonia determinations an amine odor could be detected.

Separation of the bases was carried out as described by Vickery (*46*) adding Ag_2SO_4 in boiling saturated aqueous solution until a brownish yellow precipitate was obtained, when a drop of the solution was added to a drop of saturated $Ba(OH)_2$ solution. The purine precipitate was decomposed with H_2S, and the filtrate from the Ag_2S, after removal of SO_4, was found to contain 0.317 gm. of nitrogen. Picric acid, in saturated aqueous solution, was then added until precipitation was complete. The dry weight of the picrate thus obtained was 0.984 gm., and its melting point was 278° C. (uncorrected). Picric acid was removed from the above picrate and the purine converted into the sulphate, as described by Vickery and Leavenworth (*47*). Crystals of the sulphate formed, but these were small. Picric acid was then added to the solution of the sulphate, and a picrate melting point 281° C. was obtained. This is the melting point of adenine picrate, and thus 0.199 gm. of nitrogen as adenine was accounted for.

ARGENINE FRACTION

The filtrate and washings from the purine precipitate were saturated with finely divided baryta. A buff-colored precipitate formed. The precipitate was decomposed with H_2S, and H_2SO_4 in the filtrate from the Ag_2S was exactly removed. The filtrate from the $BaSO_4$ was found to contain 0.546 gm. of nitrogen. Arginine nitrogen in this fraction was determined by the method of Van Slyke (*43*), using the apparatus devised by Holm (*19*). This gave an arginine nitrogen content of 0.156 gm.

LYSINE FRACTION

Silver was removed as Ag_2S from the filtrate and washings from the arginine fractions. Sulphate was then removed quantitatively with $Ba(OH)_2$. The filtrate from the $BaSO_4$ was found to contain 2.245 gm. of nitrogen. Lysine was precipitated from this solution by the method of Winterstein (*49*). The precipitate of the mercury salt thus obtained was centrifuged off and the filtrate found to contain only 0.202 gm. of nitrogen. The mercury salt was decomposed with H_2S, after being suspended in dilute H_2SO_4. The sulphate ion was then removed quantitatively, and the filtrate from the $BaSO_4$ concentrated to small volume, when small, yellow, rectangular crystals formed in abundance. Chlorides were then removed quantitatively with Ag_2SO_4, the excess silver removed as Ag_2S, and H_2SO_4 in the filtrate from the Ag_2S exactly removed. The filtrate and washings from the $BaSO_4$ were found to contain 1.797 gm. of nitrogen. This was concentrated to small volume, brought to the sensitive point with absolute alcohol, and a saturated solution of picric acid in ab-

solute alcohol was added until precipitation was complete. A total of 3.241 gm. of lysine picrate decomposing characteristically at 250° to 253° C. were obtained. This accounts for 0.254 gm. of nitrogen.

NONBASIC FRACTION OF THE NEUBERG PRECIPITATE

Phosphotungstic acid was removed from the filtrate of the bases from the Neuberg precipitate. The filtrate and washings from the barium phosphotungstate were concentrated and SO_4 removed quantitatively. The filtrate (solution 19) from the $BaSO_4$ on analysis was found to contain the following:

	Grams
Total nitrogen	3. 209
Ammonia nitrogen	0. 042
Amino nitrogen	2. 491
Organic solids	27. 379

The remainder of solution 19 was concentrated to small volume, when 0.069 gm. of crystalline material separated. Under the microscope the crystals were found to be large needles which were not collected in sheaves, as in the case of tyrosine, and, furthermore, the crystals were lemon yellow in color, and, dissolved in alkaline solution, gave a light green solution. The melting point was 348.8° C. (corrected). The melting point approximates that of apigenin or myricetin (*34*) but the other physical properties do not correspond. Some of the material was fused with sodium, and the Prussian-blue test found to be negative as well as the test for reduced sulphur with sodium nitroprusside. The crystals were only slightly soluble in acid, but were soluble in alcohol. It is believed that this material is a flavone or flavonol.

DIBASIC ACID FRACTION

The filtrate from the 0.069 gm. of crystalline material was concentrated to a sirup and dibasic acids removed by the method of Foreman (*11*), but varied according to the method of Kingston and Schryver (23), precipitating the dibasic acids as the barium instead of the calcium salts. The dibasic acid fraction so precipitated, after removal of barium contained 2.311 gm. of nitrogen. Beta hydroxy glutamic acid (*7*, *8*) was isolated from this fraction as the copper salt, precipitating it from aqueous solution with alcohol On analyzing this salt the following data were obtained:

	Per cent	Percentage calculated for β OH glutamic acid
Copper content found	27. 78	28. 3
Total nitrogen found	6. 50	6. 23
Amino nitrogen found	6. 17	6. 23

A total of 31.639 gm. of the copper salt were actually isolated. This contained 1.972 gm. of nitrogen, corresponding to a weight of 22.96 gm. of the β hydroxy glutamic acid, which is 0.92 per cent of the original (2,500 gm.) pollen sample.

MONOAMINO—MONOCARBOXYLIC ACID FRACTION

Carbon dioxide was passed into the alcoholic filtrate from the precipitate of the barium salts of the dibasic acid fraction. This was carried out at 0°C., according to the directions of Kingston and Schryver (*23*). From the carbamino precipitate soluble in cold

water, 0.113 gm. of crystals in characteristic sheaves were obtained. The crystals gave Millon's reaction and contained 7.64 per cent nitrogen. These crystals were, therefore, tyrosine, as the theoretical nitrogen content of tyrosine is 7.74 per cent.

DISCUSSION

The heat-coagulable fraction of the aqueous extract from the pollen contained only about 10 per cent of nitrogen, whereas albumin is generally considered to contain about 16 per cent. Likewise, the glutelin fraction contained scarcely 10 per cent of nitrogen. Accordingly, these fractions are either highly contaminated with nonprotein material or else belong to the glycoproteins.

It is evident that corn pollen contains very little, if any, globulin. Extraction with salt solution failed to give any more heat-coagulable nitrogen-containing material than extraction with distilled water; and extraction of the glutelin fraction with salt solution gave only a negligible amount of nitrogen.

The use of Neuberg's reagent in precipitating amino nitrogen proved quite disappointing. This is the second experience in which this reagent has seemed to be instrumental in decreasing amino nitrogen, probably catalyzing ring formation. In clear solutions where the end point is easily observed, this reagent no doubt can be satisfactorily employed; but it is very evident that under some conditions it is not at all satisfactory.

Since Kingston and Schryver have shown that the barium salt of inactive aspartic acid is quite insoluble, it would seem more advisable to remove the dibasic acid fraction before precipitating the bases with phosphotungstic acid; for on making the filtrate from the bases alkaline to phenolphthalein with $Ba(OH)_2$ the inactive aspartic acid may be lost, thus accounting for some of the nitrogen in the barium phosphotungstate precipitate. Immediate removal of the dibasic acids after hydrolysis would seemingly be preferable, for with the strong acids and bases present, in concentrating to small volumes, some interaction is inevitable. This viewpoint is supported by the fact that on hydrolyzing the second time the amino nitrogen was increased 24.7 per cent, and another basic precipitate was obtained on adding phosphotungstic acid. With the early removal of the relatively strong dibasic acids the tendency for amino nitrogen to decrease would surely not be so great, especially when using Neuberg's reagent.

The 0.069 gm. of crystalline material (p. 273) is believed to be a flavone or flavonol pigment. This conclusion is drawn from the following facts: It is lemon yellow in color; nitrogen and sulphur are absent; the melting point is high; it changes color in alkaline solution; and is soluble in alcohol, but quite insoluble in acid or neutral aqueous solutions.

Beta hydroxy glutamic acid is the first dibasic amino acid ever reported as occurring in pollen of any kind. Planta states that asparagin could not be detected in the pollen of *Corylus avellana*. Schulze and Planta were unable to detect asparagin or glutamin in the pollen of Corylus or pine. Heyl, working with ragweed pollen, was unable to get copper salts to separate from the amino fraction after removal of bases. The dibasic acid fraction gave a very high

yield of β hydroxy glutamic acid as 85.33 per cent of the nitrogen of this fraction as accounted for in the copper salt actually isolated. Aspartic and glutamic acids were absent.

SUMMARY

A systematic study of the nitrogenous compounds contained in the alkali extract of corn pollen, and not precipitated on neutralization is reported.

The pollen was effectively pulverized by grinding in a porcelain ball mill.

The air-dry pollen contained 3.97 per cent of moisture and 3.6 per cent of nitrogen.

Successive extractions with distilled water, 5 per cent K_2SO_4, dilute H_2SO_4, then dilute alkali removed nitrogen to the extent of 2.49 per cent of the pollen, leaving 1.11 per cent nonextractable by the above solvents.

A substance behaving like a glutelin was isolated and was found to make up 9.9 per cent of the air-dry pollen. It contained 24.96 per cent of the total nitrogen in the air-dry pollen.

Proteolytic enzymes were not active, but active carbohydrases and a pectase were present.

Twenty-five hundred grams of air-dry pollen were extracted with dilute alkali and the filtrate neutralized with dilute H_2SO_4, when a precipitate formed. This precipitate is termed the glutelin fraction. The filtrate from the glutelin fraction, after extraction with ether, was hydrolyzed and the following substances determined:

a. 0.985 gm. of adenine picrate containing 0.199 gm. of nitrogen was isolated.

b. Arginine nitrogen was determined by the method of Van Slyke. This gave 0.156 gm. of nitrogen as arginine.

c. 3.241 gm. of lysine picrate were isolated.

d. 0.1129 gm. of tyrosine was obtained.

e. 31.639 gm. of the copper salt of β hydroxy glutamic acid were isolated. Aspartic and glutamic acids were not present.

f. 13.379 gm. of choline chloroplatinate were obtained, containing 0.608 gm. of choline nitrogen.

0.069 gm. of what is believed to be a flavone or flavonol was isolated.

On acidifying the Neuberg filtrate with acetic acid 36.453 gm. of a crystalline substance with metallic luster were obtained. This substance contained neither nitrogen nor sulphur, and is believed to be the mercury salt of the above flavonol. The identification of this compound is reserved for a later report.

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